Non-invasive sampling of Snow Leopards (*Uncia uncia)* in Phu valley, Nepal

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by
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Preface

This study was conducted under joint supervision from the Norwegian University of Life Sciences (UMB) and the University of Oslo (UiO). Field work was conducted by PhD student Rinjan Shrestha on three separate occasions between 2001 and 2004. Øystein Flagstad from UiO, and later NINA (Norwegian Institutes for Nature Research) contributed with vast amounts of experience and supervised all lab work and subsequent analyses. Nils Chr. Stenseth was the formal supervisor from UiO. Per Wegge from UMB was the initiator of the project, and contributed with invaluable insight and experience from his role as external supervisor. Thank you to the people at the Centre for Integrative Genetics (Cigene) at UMB for invaluable help and training in the early stages of the project. I would like to extend my warmest gratitude to Liv Guro Kvernstuen and Nanna Winger Steen for their patience and willingness to guide me through the lab procedures necessary for this project. Rinjan Shrestha deserves great thanks for his effort in the field. His immaculate field recordings have made the analysis process so much easier. I also need to thank my supervisors for giving me the opportunity to work with the amazing Snow Leopards.

I want to thank all my family and friends for the support and positivity they have given me throughout this process. And last, but definitely not least, I need to thank Øystein Flagstad, who has been available for guidance and help in every step of the way in this project.

Marte,

Blindern, October 15th 2006.

Abstract

Snow leopards (Uncia uncia) inhabit the remote mountain ranges of the Himalayas, and are currently listed as Endangered. Due to their inaccessible habitat and sparse distribution it is difficult to compile exact estimates of the population size, gene flow, and dispersal distances, and such data are is still lacking in parts of this elusive species' range. This study showed that non-invasive tracking of snow leopards using faeces as the source of DNA is a promising method for obtaining these population parameters. Extraction success and genotyping quality were much higher for samples stored in ethanol as compared to air dried and dry stored samples. Whereas only 25% of the dry samples gave DNA of sufficient quality to be genotyped, 50% of the ethanol-preserved samples worked well. A success rate of 50% is comparable to many other studies using faeces as the source of DNA. Unfortunately, none of the applied sex markers yielded consistent results for reliable sex determination. Nine different individuals were detected among the 22 successfully genotyped samples, which may be considered a minimum estimate of the population seize. Five of these individuals likely constitute a family group with a resident male, a resident female and three cubs. The rest of the observed individuals were likely drifting animals, attracted to the valley during the mating season. This is quite high, but is explained by both the current livestock depredation rate, and the potential for the wild prey population to support a snow leopard population of 9 – 15 animals. Based on these findings we suggest that Phu valley is a snow leopard hotspot, and as such deserves increased conservation focus with proper management plans put into place. A jackknife-based capture-recapture estimate suggests a population size of 13 snow leopards (95% confidence interval [CI] = 9 – 18). This is quite high, but is explained by both the current livestock depredation rate, and the potential for the wild prey population to support a snow leopard population of 9 – 15 animals. Based on these findings we suggest that Phu valley is a snow leopard hotspot, and as such deserves increased conservation focus with proper management plans put into place.

Contents

1 Introduction

The snow leopard (*Uncia uncia*) inhabits the largest collection of high-altitude ecosystems in the world (Hunter & Jackson 1995). Its 3 million square kilometer range encompasses mountain ranges in 13 central Asian countries: Tibet, China, Nepal, India, Bhutan, Afghanistan, Pakistan, Tajikistan, Kazakhstan, Kyrgyzstan, Kazakhstan, Mongolia and Russia. However, divisions among these mountain ranges create an exceptionally fragmented and fragile population (Hunter & Jackson 1995), and despite the relatively large range, the estimated occupied range covers only 1,8 million square kilometers, mainly (60%) in Tibet and other adjoining parts of China (Jackson 2002). Snow Leopards are currently listed as "Endangered" by the IUCN Red Data Book, (IUCN 2004), and they are also banned as an Appendix I species under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Snow leopards are closely associated with arid and semi-arid shrublands, grassland or barren habitat, in the alpine and subalpine zone. They inhabit elevations ranging from 900 m to elevations exceeding 5,500 m, although mainly found between 3000 m and 4,500 m (Jackson 1996). The density of snow leopards in the wild has been observed to range from 0.4 – 0.6 adult animals per 100 km^2 in central Ladakh and the Kulu-Manali area to the south of the main Himalayan range, to 4.8 – 6.7 adult snow leopards in the Manang area of northern Nepal (Jackson 1996). Due to the remoteness of its range, its sparse distribution and low densities, it is difficult to compile accurate estimates of the wild population, and up until the last fifteen years, almost all knowledge about snow leopards in the wild accrued from anecdotal observations made from big game hunters or local herders (Jackson 1996). Today most scientists agree that an estimated global population of 3000-7000 animals remain in the wild in addition to some 600-700 Snow Leopards that survive in zoos around the world (IUCN 2004).

In Nepal, the distribution of the snow leopard seems to be concentrated in the western half of the Nepal Himalayan chain along the Tibetan border (Dhungel 1994), although they may also be found in the eastern half of Nepal (Kattel & Bajimaya 1995).

Figure 1. *Distribution of Snow Leopards throughout the Himalayan mountain range.*

The snow leopard is one of the least known and most threatened wild-ranging animals in Nepal (Dhungel 1994), with an estimated national population of 150-300 animals (Jackson *et al*. 2002). The livestock sector contributes to 65% percent of Nepals' Gross Domestic Product, and constitutes an essential element of the country's subsistence farming systems. In the diverse human communities occupying the Himalayan zone, pastoralism often constitutes the dominant livelihood amidst a conflict with the snow leopard (Jackson *et al*. 1996). These high-altitude pastures are of critical importance to the local herders, and many alpine pastures are located largely or entirely within Nepal's protected areas network (Jackson *et al.* 1996). Snow leopards are opportunistic feeders capable of killing prey up to three times their own weight (Jackson 1992). They will kill domestic livestock over most of their range, thereby posing a large threat to the livelihoods of local farmers, and substantially impacting local economies (Oli 1994a). In the absence of small rodent items from their diet, snow leopards would be expected to kill about 30

adult blue sheep (*Pseudois nayaur*) annually. Assuming an average consumable weight of 37 kg per adult blue sheep, an adult snow leopard would be expected to consume 1200 kg of large prey each year, including meat and inedible body parts (25%). A female with dependent cubs would require correspondingly more (Jackson *et al.* 1996). However, given that small rodent items occur relatively frequently in Phu Valley, an annual kill rate of 20 to 25 large ungulates might be more realistic. The negative public attitude that develops towards snow leopards when they depredate on livestock in heavy numbers, often causes herders to kill snow leopards in retaliation (Oli 1994a). Nevertheless, there have been no recorded incidences of snow leopards being killed due to poaching or herder retribution in the study area over the period of this study (P. Wegge, *pers. comm*.).

The main threats to snow leopards in Nepal include poaching for skin, poisoning by local farmers in retaliation for livestock losses, and habitat destruction, which negatively influences the snow leopards directly by removing important hunting cover, and indirectly by removing potential pasturages used by the Snow Leopard's primary prey species such as the blue sheep and the Himalayan tahr (*Hemitragus jemlahicus*) (Kattel & Bajimaya 1995). In order to implement appropriate conservation- and management programs for the species, there is an urgent need for data on population size, dispersal distances and gene flow among populations. Due to their sparse distribution, inaccessible habitat and low numbers, such data are still limited in a large range of their distribution (Jackson 2002). Up until now researchers have relied on indirect index methods, mainly using scrape marking frequency or counts of fresh tracks as a measure of relative snow leopard presence and abundance (Fox 1989). The rationale for using snow leopards signs in measuring the relative population abundance is founded on Ahlborn and Jackson (1988), who showed that the density of snow leopard markings along the travel routes is strongly associated with population density (Ahlborn & Jackson 1988). However, recently, promising direct methods such as camera trapping and non-invasive genetic sampling (Taberlet *et al*. 1997) have emerged to obtain population estimates. Camera trapping methods have improved drastically over the last decade, and the currently available methods allow identification of individuals based on their unique morphology. A population census can then be calculated based on the recapture frequencies of individuals (Wegge *et al*. 2004)

Non-invasive genetic sampling, i.e., the extraction of DNA and determination of DNA profiles for individual identification from shed material such as hair, feathers and faeces is another promising method for obtaining census estimates (Taberlet *et al*. 1997; Kohn *et al.* 1999; Creel *et al*. 2003; Flagstad *et al*. 2004). The number of distinct genotypes obtained provides a direct estimate of the minimum population size in the sampling area. However, it may not always be realistic to sample the entire target population. In these cases more sophisticated capture-mark-recapture models can be used to obtain a more realistic estimate of the population size (e.g., Brunham & Overton 1979; Eggert *et al*. (2003). Non-invasive genetic sampling does not require the capture of animals, and thus allows collection of samples without disturbing the target animal. It may therefore be particularly useful in studies of elusive animals living in remote areas that are difficult to access (Mills *et al*. 2000; Creel *et al*. 2003; Flagstad *et al*. 2004).

The approach has been successfully used for a range of mammalian species over the last decade, including ungulates (Flagstad *et al*. 2000; Maudet *et al*. 2004), and carnivores such as wolves (*Canis lupus*) (Lucchini *et al*. 2002; Creel *et al*. 2003), wolverines (*Gulo gulo*) (Flagstad *et al*. 2004), and brown bears (*Ursus arctos*) (Taberlet *et al*. 1997; Bellemain *et al*. 2005). However, although there is no doubt about the great potential of these techniques, the quality and quantity of the extracted DNA can often be poor, sometimes resulting in unacceptable genotyping error rates. Thus, noncritical use of these methods can lead to data sets with significant error rates, which can have dramatic consequences when used to design conservation and management plans for endangered and vulnerable species (Flagstad *et al*. 2004). Genotyping error is particularly problematic for genetic census studies because one single misprinting or allelic dropout produces a false individual. This means that if the sampling is sufficiently efficient, there is a potential risk for all individuals to contribute one or more erroneous genotypes to the data set (Creel *et al*. 2003). Thus, misprinting and allelic dropouts potentially cause overestimation of the minimum of sampled animals, and thus an overestimate of the population size (Creel *et al*. 2003). In order to avoid erroneous estimates of the parameters of interest, it is imperative to run a sufficient number of genotyping replicates.

This study has two main objectives. First, I will test whether noninvasive sampling of DNA from scats is a suitable method for censusing the total numbers and sex composition of snow leopards. Under this objective I will address (i) storage (70% ethanol vs. drying in the sun), (ii) the relevance of scat age, (iii) the performance of different laboratory procedures. Second, I

will use the obtained data to estimate snow leopard abundance in an area used for grazing by both livestock and wild prey (blue sheep), and discuss their predation impact on the local livestock and primary wild prey.

2 Materials and methods

2.1 Study Area

The Annapurna Conservation Area (ACA) was established in 1992, and encompasses 266.000 km^2. This study was conducted in the Phu valley (84°15' to 84°20' East, and 28°40' to 28°50' North), which is situated in the Manang area in the northern part of ACA. The altitude ranges from 3500 to 5500 meters above sea level, providing excellent snow leopard habitat (Jackson 2002). The community in Phu valley is typically traditional as modern technology has not yet reached this remote area (Gurung & Thapa 2004). Due to the limited growing season in this altitude, and limited availability of cultivated land, the main livelihood in the valley is livestock herding and trade of herbal plants However, despite the importance of livestock herding, the animal tending systems in the valley are generally poor. During daytime, the animals graze mostly unattended by herders, who find a comfortable spot where they will spend their day, rather than continuously following the animals. At nighttime the livestock is gathered in poorly constructed corrals (Gurung & Thapa 2004)..

2.2 Samples

A total of 67 samples presumed to be snow leopard scats were collected in a 125 km^2 area on the northern border of ACA in northern Nepal, over a near four-year period, between March 2001 and October 2004. All the Snow leopards in this area are believed to originate from the same population. The sampling was done opportunistically while doing other fieldwork, mostly along trails distributed throughout the study area. However, because fieldwork was concentrated in certain parts of the area, sampling was clumped throughout the valley (Fig. 2). The samples were collected from bare ground, and their age ranged from very fresh to old, dried-out scats. The sampling was concentrated along the valley bottom, at elevations ranging from 4.000 to 5.000 above sea level, because of ease of travel and indications that the densest contribution of snow leopards in this area might be found here. The samples were collected using disposal gloves and transferred into separate paper envelopes. Upon returning to the camp, the samples were then either put out in the sun to dry on a flat surface covered by plastic (48 samples), or put in plastic cases and fixed with 70% ethanol (19 samples). Before extraction the faecal samples were transported in room temperature from Nepal to Norway, and subsequently stored at room

temperature for a time period of up to three years before DNA extraction.

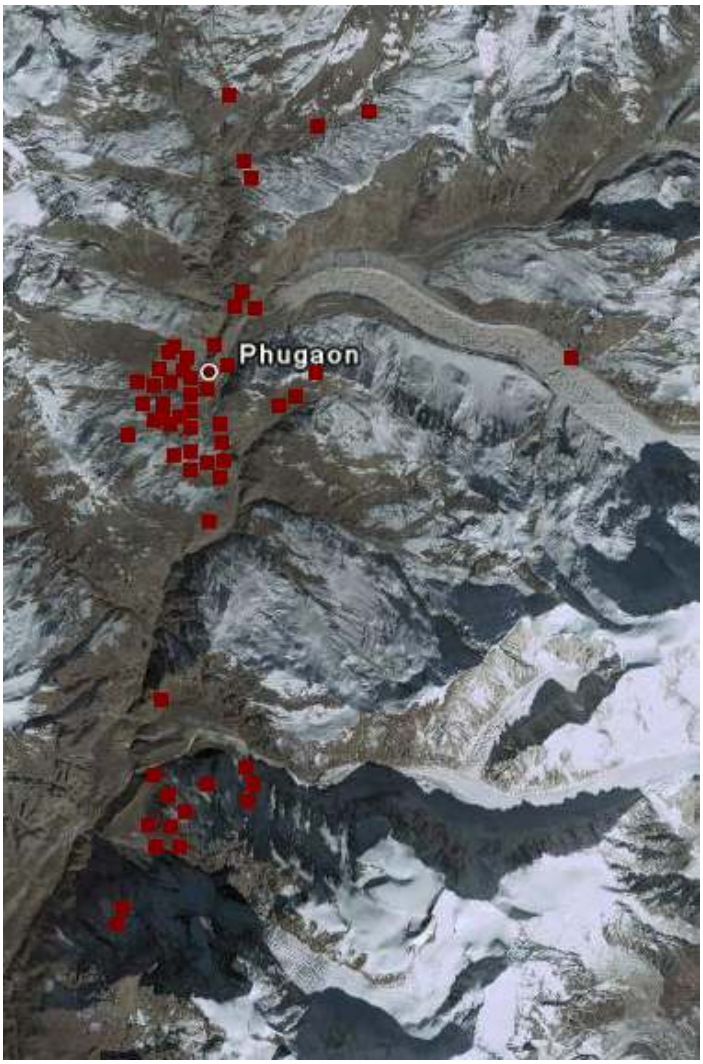

***Figure 2**: Sampling in the study area*

2.3 DNA extraction

DNA was extracted using a silica-based extraction kit, QIAamp DNA Stool Mini Kit (Qiagen, GmbH, Hilden, Germany), following a slightly modified protocol from that supplied by the manufacturer. Approximately 100 – 200 mg. were scraped off from the outside of the faeces and put in 1,5ml Eppendorf tubes, and the extraction protocol was then followed until the last step; elution of DNA. In order to increase the efficiency of the elution, the elution buffer was allowed to sit on the DNA-containing filter for five minutes instead of one minute as suggested by the manufacturer. DNA was extracted in lots ranging from five to eleven samples, and one negative

control was included in each extraction to monitor contamination. Each extraction resulted in a final yield of 200 µl for each sample, which was then stored at – 16 °C. The DNA extraction was performed in a room dedicated to extracting DNA, with pipettes dedicated to handling low copy number DNA samples such as faeces, hair, old teeth or bones.

A second DNA extraction method was tested, using MagNA Pure LC Instrument from Roche. This instrument automates physical and enzymatic purification steps, using ready-to-use nucleic acid isolation kits with prefilled cartridges. The final extracted DNA is recovered in a small volume of a special elution buffer, and can be directly used as a template for subsequent analytical procedures (Reischl *et al*. 2005). Approximately 100 mg were scraped off from the outside of the faeces and put in 2000 µl MagNA Lyser Green Beads tubes, and 600 µl lysis buffer was added. The samples were then centrifuged twice for 50 seconds at 6500 rpm., using the MagNA Lyser Instrument, in order to properly disrupt the sample cells to preprocess them prior to the nucleic acid purification. The samples were then loaded into the MagNa Pure LC Instrument together with the necessary disposables (reagent cartridges, tip trays and elution tubes from the MagNA Pure Compact Nucleic Acid Isolation Kit). I used a protocol provided by the manufacturer, originally developed for automated DNA extraction from tissue.

2.4 PCR optimization and amplification

Nuclear DNA was amplified using one marker (FCA 043) (Menotti-Raymond *et al*. 1999) that was known to work well on felids and gives strong amplification products that are clearly visible on an agarose gel. 10 µl volumes were used in all PCR amplifications. In order to optimize the amplification conditions for the microsatellite markers used, different quantities of $MgCl_2$ were tested for each marker, and the amount yielding the best result, was assigned to each marker. The quantities for all the other PCR reagents in a 10 µl reaction were: 4,92 µl H_2O, 1µl 10x PCR buffer, 3,2pmol of each primer, 0,1 mM dNTP, 0,05 µg bovine serum albumin (BSA), 0,09 µl Hot Star Taq Polymerase (Qiagen), and 2µL undiluted template. PCR conditions were pre-denaturation for 15 minutes, followed by 45 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and one minute at 72°C. A final extension step of 10 minutes at 72°C was added at the end. All samples were run in three replicates, and the amplification products were visualized on a 2% agarose gel.

A total of 15 markers were tested for use in the analysis (Table 1). Of these, 7 markers worked consistently across all samples, and were polymorph, and hence selected for the final analysis.

All samples that gave visible products in this initial test were then amplified with fluorescently labeled primers across seven additional microsatellite markers, labeled with FAM, HEX and TET. Amplifications were done using the same PCR profile as described for the test

***Table 1**: Markers tested in the initial phase of the study.*

Name	Modification	Primer sequence	Interpretable product	Heteromorph / Polymorph
FCA001	FAM	**F:** TGC TTG TCC TCT CCC TCG **R:** TGA CTG CGC CAT AGC TTT C	No	N/A
FCA008	FAM	**F:** ACT GTA AAT TTC TGA GCT GGC C **R:** TGA CAG ACT GTT CTG GGT ATG G	Yes	*Polymorph*
FCA026	FAM	**F:** GGA GCC CTT AGA GTC ATG CA **R:** TGT ACA CGC ACC AAA AAC AA	Yes	*Polymorph*
FCA031	FAM	**F:** GCC AGG GAC CTT TAG TTA GAT T **R:** GCC CTT GGA ACT ATT AAA ACC A	Yes	Monomorph
FCA043	FAM	**F:** GAG CCA CCC TAG CAC ATA TAC C **R:** AGA CGG GAT TGC ATG AAA AG	Yes	Monomorph
FCA045	TET	**F:** TGA AGA AAA GAA TCA GGC TGT G **R:** GTA TGA GCA TCT CTG TGT TCG TG	No	N/A
FCA077	HEX	**F:** GGC ACC TAT AAC TAC CAG TGT GA **R:** ATC TCT GGG GAA ATA AAT TTT GG	Yes	Monomorph
F115	TET	**F:** CTC ACA CAA GTA ACT CTT TG **R:** CCT TCC AGA TTA AGA TGA GA	Yes	*Polymorph*
FCA126	HEX	**F:** GCC CCT GAT ACC CTG AAT G **R:** CTA TCC TTGCTG GCT GAA GG	Yes	*Polymorph*
FCA272	TET	**F:** ACC TTT ACC TCC TTC CAA AAA G **R:** CAC CTT TCC ATC CAA TAA ATT C	Yes	*Polymorph*
FCA275	TET	**F:** TTG GCT GCC CAG TTT TAG TT **R:** ACG AAG GGG CAG GAC TAT CT	No	N/A
FCA391	HEX	**F:** GCC TTC TAA CTT CCT TGC AGA **R:** TTT AGG TAG CCC ATT TTC ATC A	No	N/A
FCA506	TET	**F:** AAT GACACC AAG CTG TTG TCC **R:** AGA ATG TTC TCT TCC GCG TGT	No	N/A
FCA567	TET	**F:** TCA GGG TTT TCC AGA GAA ACA **R:** TAG ACA CAT ACA GAT GGG GTG C	Yes	*Polymorph*
FCA573	HEX	**F:** TTG ACA GAG ACA GAG TGC AAG C **R:** TCT TGC AGT TGG TGA GTT TAG C	Yes	*Polymorph*

run FCA043, except for a reduced number of cycles, using 39 cycles for the FAM-labeled microsatellite markers, and 41 cycles for the TET - and HEX - labeled markers. All PCR reactions included extraction negatives, as well as positive and negative PCR controls, the positive containing template from a well-functioning sample, the negative containing water. The PCR products were then run on an ABI3730 instrument, and the subsequent allele length determination was performed with GeneMapper Software (Applied Biosystems, Foster City, CA, USA).

2.5 Determination of genotypes

Three positive PCR replicates from each sample were analyzed, using two rules; (1) An allele was only recorded if observed in two separate replicates, and (2) a homozygote was only identified if observed in three separate replicates. Thus, if none of these rules could be applied to the first analysis, another set of three replicates stemming from the same sample were analyzed.

2.6 Sex determination

Three felid-specific Y-chromosome markers were used to determine the sex of all the detected individuals (Table 2). The first sex-marker was designed from a highly conserved part of the Zn-finger gene (Pilgrim, McKelvey et al. 2005), which in mammals is found in both sex chromosomes (X and Y). This marker was developed for low-quantity DNA obtained from hair and scat samples. The Y-chromosome copy of this marker has a 3 base pair (bp) deletion, so that males are heterozygous (bp 163 and 166), and females are homozygous (bp166). All nonfelid species also produces homozygous PCR-products at bp 166, but these products are not sex-specific. This means that the marker is highly sensitive to prey contamination in the case where

***Table 2**: Primer sequences of sex identification loci used for snow leopards.*

Zn-finger	**F:**	AAG TTT ACA CAA CCA CCT GG
	R:	CAC AGA ATT TAC ACT TGT GCA
DBY7Ly2	**F:**	TCG TTA CGA CTT TCC TAG GC
	R:	CAA GGA CCC TGA CTC TTG TT
ZFLy2	**F:**	TAG TAT GTA TCC ACA GAA GT
	R:	CCT GAG ATT TTA TTC TGA CTC

result (Pilgrim *et al*. 2005). Due to this oversensitivity of this marker to faecal DNA, two additional Y-specific markers were used; ZFLy2 and DBY7Ly2. These two sex-markers are previously unpublished, but have been used extensively for sex determination in studies of the Scandinavian lynx (*Lynx lynx*), (Ø. Flagstad, *pers. comm.*). These markers will only amplify males, and positive PCR-products on agarose gel were interpreted as representing males. Two scat samples from a male snow leopard from Woodland Park Zoo in Seattle were obtained from T. McCarthy and used as a positive control in the sex determination process. Three replicates were run for each working sample for each marker, adding to a total of nine replicates for each working sample. Ten µl volumes were used in all PCR amplifications during the sex determination. The quantities for all the other PCR reagents in a 10 µl reaction, and the PCR protocols for the different markers were similar to the ones described earlier, but with 39 cycles for the Zn-finger based marker, and with 45 cycles for DBY7Ly2 and ZFLy2

2.7 Data Analysis

Genotyping errors (allelic dropouts and false alleles) were assessed for all analyzed samples. In cases where at least one (of three) replicates showed a homozygous pattern, allelic dropout was interpreted, whereas the others were considered to be heterozygous. Alleles that occurred in only one of the independent replicates were considered false alleles caused by an amplification artefact. In these cases, another three replicates were run to ensure that this was indeed the case, and not a true allele that failed to amplify in several successive replicates.

I used the procedure implemented in Gimlet (Valiere 2002) to estimate the probability of identitiy (PI); i.e. the probability that two different individuals share the same genotype. PI was estimated for unrelated individuals as well as for siblings.

Relationship analysis was performed using Lynch & Ritland's approach (Lynch & Ritland 1999) as implemented in Identix (Belkhir *et a*l. 2002), where positive mean values indicate relationship. The resulting r-value from this analysis is an index of relatedness that weighs each allele inversely by its frequency in the population, so that rare alleles are given a relatively higher weight. If a sample adequately represents a population in a Hardy-Weinberg equilibrium, the r-values obtained for full siblings, or for parent/offspring will approach 0,5 (Belkhir *et a*l. 2002). However, due to the limited number of loci included in this study, it is hard to separate between

identity by state and identity by descent. For this reason, only r-values with confidence intervals that were largely positive were interpreted as indicating a relationship. When age of the analyzed individuals is unknown, it is difficult to distinguish between parent-offspring relationships and those between full siblings if both parents haven't been sampled. Therefore, the suggestions made about parentage in this study are based purely on the assumption that the parents would have been sampled more times and over a longer timeframe than the offspring.

Population size was estimated using the jackknife approach described by Burnham & Overton (1979). This approach was originally developed to compute the species richness of an area using a definite number of successive trapping events within a strictly defined grid. However, the method may be used in special cases where the trapping events become very large, and the temporal dimension is of less importance. In this study, each successfully analyzed sample was treated as one trapping event; I then recorded how many individuals had been trapped once, twice, three times, and so on, in order to obtain a population estimate.

2.8 Livestock number and composition

The necessary data on livestock numbers and composition in the area, the number and composition of losses due to snow leopards, and the data on density of blue sheep in the area, were been obtained from R. Shrestha and P. Wegge.

3 Results

3.1 Performance of the Applied Methodology

DNA was extracted with the silica-based protocol from all 67 faecal samples collected from 2001 throughout 2004. Forty of these samples (67%) gave DNA of sufficient quality to pass the initial extraction test using marker FCA043. Excluding putatively misidentified samples that apparently originated from other species, the success rate was 63,5%. During the initial test of sample performance, I observed that samples that had been stored in 70% ethanol had a significant higher success rate than dry stored samples. The success rates were 84% (16 working samples of a total of 19 samples) for the samples stored in 70% ethanol, and 50% (24 working samples of a total of 48 samples) for the air dried samples. As an alternative extraction protocol, I used the MagNaPure LC Instrument for 37 of the collected samples. Only 13 of these samples (33%) passed the initial test for extraction success. Due to the lower success rate of this method, I chose to continue the genotyping experiments with DNA templates obtained by the silica-based protocol only.

Of the 40 working samples, 22 yielded results that were good enough to be included in the final analysis, meaning that the final success rate of the collected snow leopard samples were in fact only 33%. The reason for the exclusion of an additional 18 samples from the genotype analysis, was mainly that these amplified consistently only at one or a few loci, and was hence considered to be too low quality to be included in the final analysis. This is quite low compared to previous studies where faeces have been used as the primary source for DNA.

Allelic dropout was detected in 12,6 % of all independent replicates of inferred heterozygotes. Misprinting was much less common, and was observed in <1% of the PCR amplifications.

3.2 Estimates of Current Population Size

The probability of identity was low for unrelated individuals ($1{,}4 \times 10^{-4}$) as well as for siblings ($1{,}5 \times 10^{-2}$), strongly suggesting that two samples with identical genotypes represent the same individual. Thus, among the 22 genotyped samples, nine individuals were identified, of which all were sampled mainly in 2003 (Table 3). Only one of the samples that had been collected prior to 2003 yielded DNA of sufficient quality for genotyping. One individual (ind. 7) was sampled

over the course of 17 months, from May 2003 to October 2004, and one other individual (ind.3) was sampled twice over the course of one year, from March 2002 to March 2003. All other animals were

***Table 3**: Time of sampling for the nine identified individuals.*

Ind. #	Time of sampling
Ind.1	27.01.03
Ind.2	27.01.03
Ind.3	23.03.02, 22.03.03
Ind.4	29.03.03, 03.06.03, 04.06.03, 17.06.03
Ind.5	12.04.03
Ind.6	01.06.03
Ind.7	03.05.03, 19.06.03, 08.10.04
Ind.8	26.06.03
Ind.9	Unknown

sampled only in 2003. The nine individuals identified in the Phu Valley population in 2003 can be seen as a minimum estimate of the population size. However, 4 of these (nearly 50% of the detected animals) were sampled only once, indicating that the true population size may be higher. A capture-recapture estimate based on the observed resampling rates suggested a population size of 13 snow leopards (95% CI = 9 – 18). This gives a density of 10.4 (CI = 7,2 – 14,4) animals per 100 km^2, including cubs and offspring. Only three animals have been sampled over a longer period of time. Individual 3 has been sampled twice over the course of one year, in March 2002, and again in March 2003. March marks the last month of the mating season for snow leopards, and this may explain the apparent presence of this animal in this period only. Individual 4 has been sampled over the course of 3 months, and has been identified as a male in this study. Based on these observations, I suggest that this animal is the putative residential male in the valley. Individual 7 has been sampled in the valley over a time period of 17 months, starting early May 2003, up until the very end of the sampling period for this study. This may suggest that individual 7 is the residential female in the valley. No sampling information is available for individual 9.

3.3 Sex determination

The Zn-finger based sex determination indicated that two of the detected individuals were males

(individuals 4 and 6), whereas seven were females. Unfortunately, these results could not be confirmed by the DBY7Ly2 and ZFLy2. A male-specific band failed to amplify for all target samples, even though such a band was clearly visible for the male positive control.

3.4 Relationship Analyses

Based on the relationship analysis (Table 4), four pairs of individuals showed high relatedness values with corresponding 95% confidence intervals (C.I.) that were largely positive (Table 4). Several other pairwise comparisons also showed a positive relatedness value, but in these cases the 95% C.I. was considered to be too large for certain relationship assessment. The four pairs with the highest relatedness value and narrowest C.I. were assumed to represent either parent-offspring or full-sibling relationships. In the first group of related individuals (individuals 4, 8 and 9), individual 4 is considered to be the parent (father), since this individual is known to have been present in the valley for the longest period of time. Individual 8 has only been captured once, in the end of June 2003 (Table 3), which is about the time snow leopard females give birth to litters. Unfortunately, sampling time is not available for individual 9, but given the apparent high relatedness between individuals 8 and 9, I suggest that these are full siblings. Looking at the allele composition of all analyzed individuals, individual 7 is the only possible mother of both these individuals, given that individual 4 is their father. Individual 6 is also a likely offspring of individual 7. This inference is based on the assumption that individual 7 is the residential female in the valley, as well as the observation that individual 6 was first (and only) sampled around the snow leopard birthing in 2003. Notably though, all sampled individuals could be excluded as being the father of individual 6. Yet, this individual could belong to the same litter as individuals 8 and 9, which would imply multiple paternity of this litter (Figure 3).

***Table 4**: Lynch & Ritland mean r-values and C.I. values*

	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	0,26 (-0,48 – 0,66)	0,35 (-0,30 – 1,00)	-0,45 (-0,93 – 0,07)	-0,24 (-0,66 – 0,19)	-0,38 (-0,59 – -0,10)	- 0,06 (-0,68 – 0,64)	-0,49 (-0,97 – 0,31)	-0,46 (-0,75 – 0,09)
2		-0,18 (-0,45 – 0,05)	-0,08 (-0,72 – 0,66)	-0,45 (- 0,85 – -0,27)	0,02 (-0,50 – 0,55)	-0,08 (-0,29 – 0,17)	-0,21 (-0,65 – 0,22)	-0,64 (-0,86 – 0,35)
3			0,07 (-0,29 – 0,67)	-0,07 (-0,51 – 0,44)	-0,58 (-0,81 – -0,37)	-0,62 (-0,81 – -0,50)	0,16 (-0,43 – 0,83)	0,03 (-0,39 – 0,50)
4				-0,19 (-0,65 – 0,45)	-0,36 (-0,52 – -0,17)	-0,45 (-0,71 – -0,30)	***0,40 (-0,06 – 1,00)***	***0,32 (-0,14 – 1,00)***
5					-0,10 (-0,44 – 0,50)	-0,04 (-0,56 – 0,62)	-0,28 (-0,79 – 0,24)	-0,15 (-0,58 – 0,69
6						***0,13 (-0,13 – 0,60)***	-0,07 (-0,42 – 0,43)	0,07 (-0,34 – 0,41)
7							-0,35 (-0,71 – 0,12)	-0,10 (-0,57 – 0,46)
8								***0,32 (-0,13 – 0,76)***

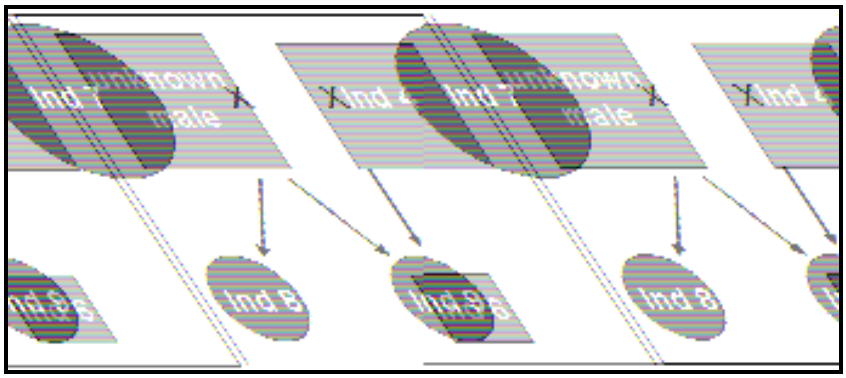

***Figure 3**: Relationships in the snow leopard population, squares marking males and circles marking females. The figure shows the three cubs in one litter, with two different fathers, an example of multiple parentage which has been observed on several occasions in felids..*

In conclusion, these results and the associated interpretations are compatible with field observations that there is one reproductive unit in the valley and that individuals 4 and 7 are the residential male and female, respectively.

3.5 Predation impact

The community in Phu valley, centered in Phu village (Phuagon), consists of 33 households with a livestock herd of nearly 2.000 animals (Table 5). This livestock grazes throughout the majority of the valley, covering a total area of 152km^2, completely overlapping with the 125km^2 blue sheep grazing area in the valley, which is also where the sampling was done (P. Wegge, *pers. comm*.).

***Table 5**: Livestock numbers and predation rates in Phu Valley*

	Livestock (average 2002 - 2003)	**Animals predated by snow leopard** (average 2001 - 2003)	**Percentage predated**
Yak	767	16..3	2,13 %
Goats	650	41.3	6,35 %
Sheep	372	17..6	4,73 %
Cows	106	0.3	0,28 %
Horses	75	0.7	0,93 %
Total	**1970**	76.2	**3,87 %**

The annual livestock depredation rate in the valley is 3,87% (Table 5), which is considerably higher than the depredation rate of 2,6% reported by Oli in the neighbouring Ngishyan Valley (Oli 1994b).

A total count of the blue sheep population in Phu Valley in 2003 yielded a population of 1054 animals (P. Wegge, *pers. comm*.). With prey consisting of 66% of a snow leopards' diet, a population this size should be able of supporting a population of 8-15 snow leopards.

4 Discussion

4.1 Performance of the applied method

Murphy *et al*. (2002) found that the most efficient faecal DNA preservation method was storage in 90% ethanol (Murphy *et al*. 2002). This is strongly supported by the results in this study, where the airdried samples performed markedly poorer than the samples that had been stored on 70% ethanol, in every step of the project. The final success rate in the study, including only the 22 samples used to obtain individual genotypes, were 52% (10/19 samples) for samples stored on 70% ethanol, and 25% (12/48 samples) for airdried samples. Increased success rate of samples is important because more samples can be identified to individuals, and because it significantly reduces the number of PCR reactions needed in order to obtain acceptable results (Murphy *et al.* 2002). It was not possible in this study to find the relevance of scat age on success rate, since all but two of the successfully amplified samples were sampled during the first half of 2003, thus not leaving any grounds for comparison between the samples.

The genotyping error rates obtained in this study are comparable to those observed in Flagstad *et al*. (2004), with a slightly higher allelic dropout rate (12,6% vs. 9,8%), and an equally low rate of misprinting. The comparable error rates and the same rigid use of predefined criteria for accepting single-locus genotypes strongly suggest that most errors were eliminated from the present data set prior to the final analysis. This was a critical task in this study, since even a minor error rate in single-locus consensus genotypes would lead to an upward bias of a capture-recapture estimate of population size based on more than a few loci (Flagstad *et al*. 2004)

DNA-extracts stemming from field-collected faeces often yield small amounts of target DNA, and may also contain polymerase chain reaction (PCR) inhibitors, so that microsatellite genotypes from noninvasive samples are affected by false alleles (misprinting), producing false heterozygotes, and failure of alleles to amplify (allelic dropout) (Creel *et al*. 2003) producing false homozygotes (Flagstad *et al*. 2004). Thus, misprinting and allelic dropouts potentially cause over-estimation of the minimum number of sampled animals, and thus an overestimate of the population size (Creel *et al*. 2003). However, these are recognized problems, and several authors have developed methods to address them, including dropping poor-quality samples from the data set, or to accept that genotyping errors never will be completely eliminated, and analyze

the data with a 'matching approach', where a mismatch between two samples at a locus indicates that they either came from two different individuals, or that they stem from the same individual, with a genotyping error (Creel *et al*. 2003). Since the matching approach does not require the complete elimination of errors from noninvasive genotypes in order to produce unbiased estimates of population size, it promises to be an accurate and efficient method of applying noninvasive genetic data to population estimation (Creel *et al*. 2003). Applying these methods, I mean to have reduced this risk to a minimum.

There might be several reasons for the low success rate of the collected samples (33%); (1) All samples were collected on bare ground, which in earlier studies has been found to be a reason for decreased success rates (e.g. Flagstad *et al*. 2004). (2) Most samples had been stored for up to three years at the time of DNA extraction. (3) The majority of the samples were stored dry instead of on 70% ethanol. (4) The inhibitor-binding substances present in the applied extraction kit, and the BSA used during amplification, may not have efficiently removed the PCR inhibitors. (5) The Hot Star Taq. Polymerase used may not have been efficient enough in amplifying the low-copy-number DNA found in snow leopard faeces. There is definitely room for improvement of the current method, both in terms of sampling and storage, and in terms of better suited extraction reagents and enzymes.

4.2 Sex determination

Although the Zn-finger showed two males in the sample area, the fact that neither DBY7Ly2 nor ZFLy2 yielded any males, means that we cannot be certain about how many males there are in the target population, or even if there are any. The fact that prey species DNA will tend to dominate in all amplifications using Zn-finger, subsequently producing false females, means that this marker cannot be used as a certain indication of males, but rather as a suggestion. As for DBY7Fly2 and ZFLy2, it seems that these markers require high-quality DNA in order to work properly, as the positive sample, that was considered to be of much higher quality than the project samples, yielded positive results every time, whereas none of the project samples amplified as males. The reason for the higher quality of the positive sample was that (a) it was fresh when sampled, not lying on the ground for more than a few hours at the most, whereas the project samples potentially could have been lying on the ground for several weeks, (b) the storage period for the positive was approximately two weeks, whereas the project samples had been stored anywhere between 3 and 4 years at the date of DNA isolation, and finally, (c) the positive sample

was stored on 80% ethanol, whereas the majority of the project samples were airdried and stored dry.

In studies involving DNA extraction from snow leopard faeces, there is never any guarantee for being able to collect only fresh samples, and most of the time the storage period after sampling will exceed the two weeks that was the case for the well- functioning positive samples in this project. Hence, the methods used for sex determination of snow leopards need considerable improvement, as those available today are insufficient to conclude with certainty the number of males in a population. As a result of these findings it can only be suggested that there are two males in the population, although further testing with new and improved methods will be needed in order to verify this.

4.3 Family groups

It is known that at least one family group resides in the study area (P. Wegge, *pers. comm*.). As the ages of the individual snow leopards aren't known, it was not possible to assign à priori what individuals were parents and what individuals were offspring, so suggestions could only be made based on the temporal presence of the samples. This means that if an individual has been sampled over a longer time period, and the first sampling occasion happened earlier than that of another individual, one might suggest that this individual is the actual parent. However, these will only be loose suggestions, and further tests will be needed in order to say anything with a high degree of certainty.

The presence of a family group in the valley is supported by the findings of this study. Comparison of the genotypes of all the sampled animals who that, of all the identified animals in the population, individual 7 is the only one that is compatible as mother for both individuals 8 and 9, given that individual 4 is the father (Table 4). The relatedness value and its associated confidence interval can neither confirm nor reject this, but the indications that individuals 4 and 7 are the residential animals in the valley, support this interpretation. The last likely offspring of the putative residential female, individual 6, carries a genotype that excludes individual as a possible father. An unsampled male must therefore have sired individual 6. Given that drifting males likely are attracted to the valley during the mating season, several of them would probably attend to mate with the residential female. Individuals 6, 8 and 9 could therefore belong to the same litter, which would be an example of multiple paternity. This phenomenon is well documented in

felids (Say *et al*. 1999; Jewgenow *et al*. 2005).

4.4 Population size

The low probability of identity strongly suggested that individuals could be reliably distinguished from the 7 loci used in this study. Nine different individuals were detected in the Phu Valley during the course of the study, indicating that a minimum of nine snow leopards were present in the valley in spring 2003. The capture-mark-recapture estimate suggested that an additional four animals likely were present at this time (point estimate = 13, 95% C.I. = 9 - 18 individuals). One potential problem with using the jackknife approach described by Burnam & Overton (1979) for obtaining a population estimate is the implicit assumption of population closure, which is violated in the case of this study, and that may cause an upward bias of the population estimate. However, given that we with a high degree of certainty have not been able to sample the entire population in Phu Valley, and having obtained a minimum population estimate of 9 individuals, a population estimate of 13 animals seems reasonable.

From an ecological point of view, a population density of 13 animals or 10.4 snow leopards per 100 km^2 seems rather high, as most similar studies have found densities ranging from 0.4 - 0.6 snow leopards per 100km2, to 4,8 - 6.7 snow leopards per 100km^2 (Jackson 1996). However, Jackson (1996) found a similar population density in Langu Valley in Nepal, suggesting that such high population densities in certain hotspots are certainly possible. The population in Phu valley lacks closure, receiving drifting individuals from neighbouring valleys, as was confirmed by the high degree of unrelatedness by several of the individuals in the population. Several of the identified individuals have been sampled only on one occasion or on several occasions within the same day, during the first three months of 2003. This time period marks the mating period, when mature, drifting males will tend to seek out areas with resident mature females. Unfortunately it was not possible, with the methods available, to determine whether or not the snow leopards that had only been sampled on one day, were males. Whatsoever, it seems likely that the population estimate is elevated due to the presence of drifting males seeking mature females in the valley during the sampling period.

The grasslands of Phu Valley have been used for centuries by the local inhabitants for livestock grazing. Currently, the livestock population (1970 animals) in the valley is approximately twice as big as the blue sheep population (1054 animals). The relatively high depredation rate (3,87%) in the valley compared to the neighbouring valley, supports the finding

of a big snow leopard population. In addition, Oli (1994) found that livestock contributed 34% of the Annapurna area snow leopard's diet, compared to 66% for wild prey (Oli 1994b). With wild prey consisting of 66% of a snow leopard's diet, a bharal population the size of the one found in Phu Valley should be capable of supporting 8 – 15 snow leopards at a 13 percent harvesting rate. This is highly consistent with the population estimate of 9 – 18 animals obtained using the jackknife approach described by Burnham & Overton (1979).

5 Conclusions and management implications

The results in this study show that, despite a relatively low success rate for snow leopards, non-invasive genetic sampling is a suitable method for identifying individual snow leopards, for censusing the numbers of snow leopards in a population, and in time possibly also to determine with certainty the gender composition in a snow leopard population. In order to maintain the best quality possible, faecal samples should be stored on at least 70% ethanol, and the storage period between sampling and DNA extraction should be minimized. For sex determination to be successful, the samples apparently need to be relatively fresh, which is often not obtainable within the frames of snow leopard studies. New and improved methods therefore need to be developed, as those available today often are insufficient to conclude with certainty the gender composition in a snow leopard population.

The Phu valley snow leopard population consists of 9 -18 animals. The population lacks closure, receiving drifting animals from neighbouring valleys, which might tend somehow elevate the obtained population estimate. There is, however, a confirmed population minimum of 9 individuals, of which 5 most likely belong to one reproductive unit consisting of the residential male and the residential female. This population census is also confirmed by livestock predation data, and bharal population data, which is sufficiently large to support a population of 9 -15 individuals.

From a management point of view, the observation that the Phu valley snow leopard population is relatively large, indicates that the area is a hotspot, with optimal snow leopard habitat, and few disturbing external factors. As a large part of the valley is used by local herders for grazing their livestock, it may be important to devote attention to developing herding procedures and educational programs, in order to increase the understanding and awareness of the locals towards snow leopards. The finding that Phu valley may be a snow leopard hotspot is important in terms of snow leopard conservation, and may provide a basis on which to develop a management plan in the future.

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Appendices

Appendix 1 – Raw data for the 22 genotyped faecal samples

With consensus genotype C#

	FCA008		FCA026		F115		FCA126		FCA272		FCA567		FCA573	
1	128	131	148	151	196	196	141	152	112	116	91	91		
1	128	131	148	151	196	196	141	141	112	116	91	91		
1			148	151	196	196	141	141			91	91		
1			148	151			141	141			91	91		
C1	128	131	148	151	196	196	141	141	112	116	91	91		
10	131	135	148	148	199	199	141	141	116	116	91	93	190	194
10	131	135	148	148	199	199	139	141	116	116	91	93	190	194
10	131	135	148	148	196	199	141	141	116	116	91	93	190	190
10					196	196	141	141					190	194
10					196	196								
10					196	199								
C10	131	135	148	148	196	199	141	141	116	116	91	93	190	194
12	128	131	148	151	199	202	141	152	112	116	91	91	190	190
12	128	131	148	151	199	202	141	152	112	116	91	91	190	190
12	128	131			202	202	141	152	112	116	91	91	190	190
C12	128	131	148	151	199	202	151	152	112	116	91	91	190	190
14	131	135	148	151	199	202	152	152	112	116	91	93	190	194
14	131	135	148	151	199	202	152	152	112	116	91	93	190	194
14	131	135	148	151	202	202	152	152	112	116	91	93	190	194
14													190	194
14													190	194
14													190	194
C14	131	135	148	151	199	202	152	152	112	116	91	93	190	194
18	128	131	148	151	196	196	141	152	112	116	91	91	190	190
18	128	131	148	151	195	197	141	152	112	116	91	91	190	190
18	128	131	151	155	195	197	141	152	112	116	89	91	190	190
18	128	131	151	155	194	199	152	152	112	116	89	91	190	194
C18	128	131	148	151	?	?	141	152	112	116	89	91	190	190
23	128	131	151		194	199	152	152	112	116	89	91	190	194
23	128	131	151		194	199	152	152			89	91	194	194
23					194	199	152	152			89	91	194	194
C23	128	131			194	19	152	152	112	116	89	91	190	194

	FCA008		FCA026		F115		FCA126		FCA272		FCA567		FCA573	
26	131	135	148	151	199	202	152	152	112	116	91	93	190	194
26	131	135	148	151	199	202	152	152	112	116	91	93	190	194
26	131	135			199	202	152	152	112	116	91	93	190	194
26													190	194
26													190	194
C26	131	135	148	151	199	202	152	152	112	116	91	93	190	194
28	131	135	148	148	194	202	141	152	116	116	89	91	194	194
28	131	135	148	148	194	202	141	152	116	116	89	91	194	194
28	131	135	148	148	194	202	141	152	116	116	89	91	194	194
28			148	148	194	202								
28			148	148										
28			148	148										
C28	131	135	148	148	194	202	141	152	116	116	89	91	194	194
29	131	135	148	151	199	202	152	152	112	116	91	93	190	194
29	131	135	148	151	199	202	152	152	112	116	91	93	190	194
29	131	135	148	151	199	202	152	152	112	116	91	93	190	194
C29	131	135	148	151	199	202	152	152	112	116	91	93	190	194
31	131	135	148	151	196	196	152	152	112	116	91	93	190	194
31	131	135			199	202	152	152	112	116	91	93	190	194
31	131	135			199	202	152	152	112	116	91	93	190	194
31					199	202	152	152						
31					199	202	152	152						
C31	131	135	148	151	199	202	152	152	112	116	91	93	190	194
35	131	135	148	155	196	196	141	152	114	116	89	89	194	194
35	131	131	148	155	196	202	141	152	114	116	89	91	194	194
35	131	135	148	155	196	202			114	116	89	91	194	194
35	131	135	148	155	196	202			114	116	89	91		
35	131	135	148	155										
35			148	155										
35			148	155										
35			148	155										
35			148	155										
C35	131	135	148	155	196	202	141	152	114	116	89	91	194	194

	FCA008		FCA026		F115		FCA126		FCA272		FCA567		FCA573	
37	131	135	148	155	196	196	141	152	116	116	89	89		
37	131	135	148	155	196	196	152	152	116	116	89	91		
37			148	155	196	196	141	141	116	116	89	91		
37			148	155	196	202	147	149			89	91		
37					196	202	147	149						
37					196	202								
37					196	202								
C37	131	135	148	155	196	202	141	152	116	116	89	91		
W3	131	135	148	148	194	202	141	152	116	116	89	91	194	194
W3	131	135	148	148	194	202	141	152	116	116	89	91	194	194
W3	131	135	148	148	194	202	141	152	116	116	89	91	194	194
W5	131	135	148	151	199	202	152	152	112	116	91	93	190	194
CW3	131	135	148	148	194	202	141	152	116	116	89	91	194	194
W5	131	135	148	151	202	202	152	152	112	116	91	93	194	194
W5	131	135			202	204	152	152	112	116	91	93	190	190
W5					204	204	152	152					190	194
W5					199	199	152	152					190	194
W5					199	199	152	152					190	194
w5					196	199	152	152						
w5					196	202								
w5					196	202								
w5					196	202								
CW5	131	135	148	151	199	202	152	152	112	116	91	93	190	194
W6	131	135	148	151	199	202	152	152	112	116	91	93	190	194
W6	131	135	148	151	199	202	152	152	112	116	91	93	190	194
W6	131	135	148	151	199	202	152	152			91	93	190	194
CW6	131	135	148	151	199	202	152	152	112	116	91	93	190	194
W7	131	135	148	155	196	202	141	152	114	116	89	91	194	194
W7	131	135	148	155	196	202	141	152	114	114	89	91	194	194
W7	131	135	148	155	196	202	151	152	116	116	89	91	194	194
w7									114	116			194	194
CW7	131	135	148	155	196	202	141	152	114	116	89	91	194	194

	FCA008		FCA026		F115		FCA126		FCA272		FCA567		FCA573	
W8	131	135	146	151	199	199	152	152	116	116	91	91		
W8	131	131	148	148	199	199	152	152	116	116	91	91		
W8	131	131	148	151	199	202	152	152	116	116	93	93		
W8	131	131			199	202	152	152	116	116	91	91		
W8	131	135			202	202					91	91		
W8	131	131			202	202					91	91		
W8	135	135			202	202					91	91		
W8					202	202								
CW8	135	135	148	151	199	202	152	152	116	116	91	91		
A	135	135	148	151	202	202	152	152	112	116	89	91	190	190
A	131	135	148	151	202	202	152	152	112	112	89	91		
A					202	202	152	152	116	116				
A					202	202								
CA	131	135	148	151	202	202	152	152	112	116	89	91		
B	128	131	148	151	199	202	141	152	112	116	89	91	190	190
B	128	131	148	151	199	202	141	152	112	116	91	91	190	190
B			148	151	199	202	141	152	112	116	91	91	190	190
B							141	152			89	91		
B											89	91		
B											89	91		
CB	128	131	148	151	199	202	141	152	112	116	89	91	190	190
E			148	148	194	202	141	152	116	116	89	91		
E							141	152			89	89		
E	131	135	148	148	194	202	141	152	116	116	89	91	190	194
E	131	135	148	148	194	202	141	152	116	116	89	91		
E							141	152			89	89		
E							141	152			89	89		
CE	131	135	148	148	194	202	141	152	116	116	89	91		
G	131	135	148	151	199	202	152	152	112	116	89	91	190	194
G	131	135	148	151	199	202	152	152	112	116	89	91	190	194
G	131	135	148	151	199	202	152	152	112	116	89	91		
G					199	202	152	152						
G							152	152						
G							152	152						
CG	131	135	148	151	199	202	152	152	112	116	89	91	190	194

	FCA008		FCA026		F115		FCA126		FCA272		FCA567		FCA573	
I	128	131	148	148	199	202	141	141	112	116	89	91	190	190
I	128	131	148	151	202	202	141	152	112	116	89	91	190	
I	128	131	151	151	194	202	141	152	112	116	91	91	190	
I			148	151	194	202	141	152	112	112				
I			148	151	194	202			112	112				
I			148	148					112	112				
CI	128	131	148	148	194	202	141	152	112	116	89	91	190	